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Suppression of calbindin D28K in estrogen-induced hamster renal tumors

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Abstract

It has been hypothesized that generation of reactive estrogen–quinone species and oxidative stress, both of which result from the metabolic activation of estrogens, plays an important role in estrogen-induced carcinogenesis. In the present investigation, we used an estrogen-induced hamster renal tumor model to identify gene(s) associated with oxidative stress that may be differentially expressed in estrogen-induced tumors compared with untreated controls. Hamsters were implanted with 17β-estradiol (E2) for 7 months. This treatment resulted in the development of target organ specific kidney tumors. Delta differential PCR technique on RNA isolated from estrogen-induced hamster renal tumors and untreated control kidneys identified a number of cDNA fragments that were differentially expressed in tumor RNA compared with untreated controls. We report the cloning of one of the differentially expressed cDNA fragments, the hamster calbindin-D28k (Cb28k) cDNA, and present a finding that both Cb28k mRNA and protein are suppressed in estrogen-induced hamster renal tumors compared with untreated controls. Cb28k is a Vitamin D3-dependent calcium binding protein that acts as a buffer to maintain intracellular calcium homeostasis, although its exact role is still not clear. Since Cb28k gene has been shown to be associated with providing cells resistance against oxidative stress, Cb28k may be an important biomarker in estrogen-mediated carcinogenesis and oxidative stress.

Keywords: Calbindin; Hormonal carcinogenesis; Renal tumors; Oxidative stress

1. Introduction

Prolonged exposure of women to estrogens has consistently been associated with an increased risk of cancer [1–3], and steroidal estrogens, including 17β-estradiol (E2), have been classified as human carcinogens by the National Institute of Environmental Health Sciences [4]. Recently, a clinical trial of estrogen plus progestin replacement therapy was terminated early due to elevated risk of breast cancer and lack of overall benefit to the patients [5]. In studies of mechanisms of hormonal carcinogenesis, natural hormones, such as E2, as well as synthetic estrogens, such as diethylstilbestrol, have been shown to induce tumors in rodent models [6–8]. However, the mechanisms of estrogen-induced carcinogenesis have not yet been fully characterized [7,9], though the estrogen-induced hamster kidney and the estrogen-induced rat mammary tumor models have provided a degree of understanding of the mechanism of human mammary carcinogenesis through shared biochemical and molecular characteristics [7–13]. In principle, however, the potential of an estrogen to initiate estrogen–estrogen receptor mediated pathways does not always correlate with its carcinogenic potential in rodent models [7,14–16]. It has been suggested that oxidative stress resulting from metabolic activation of carcinogenic estrogens plays an important role in the estrogen-induced carcinogenic process [7,17–20]. Recent studies of the hamster renal tumor model have shown that sub-chronic treatment of hamsters with the carcinogenic estrogen E2 results in increased oxidant stress levels in the target organ kidney, as reflected by increased isoprostane formation, whereas treatment of hamsters for the same length of time with weakly carcinogenic estrogen 17α-ethinylestradiol (EE) does not [7]. Additionally, treatment of both the hamster kidney tumor cell line H-301 and of the human breast cancer cell line MCF-7 with the carcinogenic estrogen E2 results in oxidant stress levels that are increased compared with those observed after EE treatment [17]. A possible explanation is that carcinogenic and

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noncarcinogenic estrogens differ in their metabolic activation potential to produce catechol estrogens [15,18,19]. In turn, redox cycling of catechol estrogens and estrogen quinones results in free radical formation and in increased oxidative stress [19,20].

Since E2 has high estrogenic potential, and its metabolism, unlike that of EE, confers high levels of oxidant stress [7,17] through metabolic redox cycling of carcinogenic estrogen metabolites, it plays a substantial role in estrogen-induced carcinogenesis. In the present study, we attempt to identify genes associated with oxidative stress that may be differentially expressed in estrogen-induced tumors compared with untreated controls. We identified and cloned the hamster calbindin-D28k (Cb28k) cDNA, and investigated its expression pattern in untreated control kidneys and renal tumor tissue of hamsters treated with E2 for 7 months. Cb28k is a Vitamin D3-dependent calcium binding protein that has been suggested to act as a buffer in maintaining intracellular calcium homeostasis, but its exact role is still unclear [21,22]. However, the presence of Cb28k gene in cells appears to be associated with greater resistance to oxidative stress [23,24]. We present a finding that both Cb28k mRNA and protein levels are suppressed in estrogen-induced hamster renal tumors compared with untreated controls. Cb28k may, therefore, be an important biomarker relevant in studies estrogen-mediated carcinogenesis.

2. Methods

2.1. Treatment of animals

Male Syrian hamsters (4–6 weeks old, Harlan Sprague-Dawley, San Diego, CA) were housed at our animal facility with Purina Rodent chow and water available ad libitum. Hamsters were sub-chronically treated with E2 (25 mg, s.c.) implants for 7 months as described previously [7,25]. A control group was sham operated but received no treatment. Hamsters received a second estrogen implant after 3 months of initial treatment [7,25]. After 7 months of continuous estrogen treatment, hamsters were sacrificed, and kidneys from untreated controls and kidney tumors from tumor-bearing kidneys were immediately excised and frozen in liquid nitrogen. E2 and other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Characterization and cloning of the hamster Cb28k gene

2.2.1. Differential display polymerase chain reaction

Total RNA was isolated from E2-induced hamster renal tumors and from untreated age-matched hamster kidneys. Messenger RNA was reverse transcribed and PCR amplified using a delta differential display kit (Clontech, Palo Alto, CA) and a sequencing gel methodology [26–29]. The delta differential display protocol uses arbitrary primers and allows for the selection of random cDNA fragments that are differentially expressed between controls and treated groups [30]. cDNA fragments that showed differential expression patterns between control kidneys and renal tumors were excised from acrylamide gels. DNA was extracted, purified, and re-amplified using the same primer sets for amplification that was initially used for differential display. These cDNA fragments were size fractionated on 2% agarose gels and visualized by ethidium bromide staining. The bands were excised from agarose gels, DNA was extracted using the Geneclean II kit (Bio 101 Inc., La Jolla, CA), and subjected to sequence analysis [31].

2.2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification

The expression of Cb28k mRNA in hamster estrogen-induced renal tumors and in age-matched untreated control kidneys was quantified by using RT-PCR amplification kit (Invitrogen, Carlsbad, CA). Primers for PCR were designed using the sequence information from the previously cloned cDNA fragment. RT was performed using 5 μg of total cellular RNA and an oligo-dT_{18} primer. One to two microliters of the RT product was used for PCR amplification with gene-specific primers. For semiquantitative comparisons, the same RT reaction products were used for PCR amplification of cyclophilin cDNA. PCR products were separated on a 1.5% agarose gel, amplicons were visualized by ethidium bromide staining, and negative films were used to record the images. Band density was measured using a Molecular Dynamics densitometer/ImageQuant program (Sunnyvale, CA), and expression of the calbindin-D28k cDNA fragment was quantified by representing the density of the appropriate band as a ratio to the amount of the corresponding cyclophilin amplified fragment [32]. Control experiments were performed to select the number of cycles for PCR amplification such that the PCR products obtained were within the exponential phase of amplification.

2.2.3. Cloning

For cloning of the full-length hamster Cb28k cDNA, primers were designed based on maximum homology between rat, mouse, human cDNA sequences, and on the partial hamster cDNA sequence that we had obtained after initial differential display PCR. Total RNA isolated from untreated hamster kidney was reverse transcribed and used for PCR amplification. Primer set 5′-CTC TCA AAC TAG CCG CTG CAC C (forward) and 5′-GAC AGA TTT CTG TGT CAC CTG TCT CCC TTC (reverse) was used for PCR that resulted in the amplification of ∼1400 bp cDNA fragment. This fragment was excised from a 1.5% agarose gel, and the cDNA was purified using a QIAquick PCR purification kit (QIAgen, Valencia, CA). The ∼1400 bp fragment was ligated into pCR4Blunt-TOPO vector (Invitrogen), and subsequently transformed into competent *Escherichia. coli* (DH5α-T1® strain). Transformants were plated on Luria–Bertrani agar plates containing kanamycin (100 μg/mL), and, following overnight incubation at 37 °C, single colonies were identi-

fied as probable positive clones [31]. These were grown in LB/kanamycin (100 μg/mL) suspension overnight at 37 °C, and the recombinant plasmids were isolated and purified using NucleoSpin Plasmid Kit (Clontech) according to the manufacturer's recommendations. Positive plasmid clones were identified by PCR amplification of the plasmid DNA using M13 (forward and reverse) viral primers, calbindin-specific primers (forward and reverse), and by digestion of the plasmid DNA with EcoRI for 1 h at room temperature followed by electrophoresis on 1.5% agarose gels to screen for the presence of the cDNA insert. The calbindin cDNA sequence information was obtained using an ABI 3100 sequencer at our DNA Analysis and Sequencing Facility. Both M13 and gene-specific primers (forward and reverse) were used in the sequencing reaction, and the final sequence was verified using regions of overlap.

2.3. Immunodetection of calbindin

2.3.1. Protein isolation

Renal tissue excised from control hamsters and from E2-induced tumors was frozen in liquid nitrogen and stored at −80 °C. Before analysis, this frozen tissue was thawed on ice in RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mL EGTA, 1% Triton, supplemented with 100 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 0.2 M Na_3VO_4, and 0.2 M NaF), and homogenized using PRO 200 homogenizer with a 5 mm × 75 mm generator (PRO Scientific, Oxford, CT). Homogenization was carried out by moving the motor speed dial of the homogenizer from 0 to 5 (0–30,000 rpm) back and forth five times, for a total homogenization time of ∼5 s. The homogenate was pelleted by centrifugation at 10,000 × *g* at 4 °C for 10 min, and the supernatant was saved for further analysis. Total protein concentration in each sample was determined using the Pierce Protein Assay Kit (Rockford, IL) according to the manufacturer's recommendations.

2.3.2. Immunodetection

Twenty micrograms of the total protein mixture isolated from triplicates of control or E2-induced renal tumor tissue was size fractionated on a 15% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane under standard conditions. After blocking in 5% dry non-fat milk/TBS/0.1% Tween-20 at 4 °C overnight, an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of calbindin of human origin (Santa Cruz, CA) was diluted 1:100 in 3%BSA/TBS/0.1% Tween-20 and used for immunodetection. After incubation for 1 h at room temperature with the primary antibody, the membrane was washed once for 15 min and twice for 5 min in TBS/2% Tween-20. HRP-conjugated bovine anti-goat IgG was diluted 1:2000 in 3%BSA/TBS/0.1% Tween-20 and used as secondary antibody. After incubation for 1 h at room temperature, the membrane was washed again as described above. Chemiluminiscent detection was performed by incubating the membrane with ImmunStar HRP substrate (BioRad) for 5 min at room temperature, and then exposing it to X-ray film for 15 s. Band intensity was then quantified using a densitometer (Molecular Dynamics). The same membrane was stripped and reincubated with glyceraldehyde-3-phoshate dehydrogenase (GAPDH) monoclonal mouse anti-rabbit antibody (Research Diagnostics, Flanders, NJ). The secondary antibody used for GAPDH detection was ECL anti-mouse IgG (Amersham, Piscataway, NJ).

3. Results

3.1. Cloning of full-length hamster Cb28k cDNA

Differential display PCR screening method enabled us to identify 19 cDNA fragments that were overexpressed and 18 cDNA fragments that were suppressed in estrogen-induced kidney tumors compared with age-matched untreated renal tissue based on at least two-fold difference upon densitometric scanning (data not shown). The inter-analysis variance between triplicate samples ranged from 25 to 35%. DNA sequencing and BLAST analysis of a short (∼350 bp) cDNA fragment, mRNA expression of which was suppressed in estrogen-induced hamster renal tumors compared with untreated age-matched control kidneys showed its partial homology to the 3′-end of mouse, rat, and human Cb28k cDNAs (data not shown). Attempts were subsequently made to PCR amplify a full-length hamster Cb28k cDNA from the kidney tissue. Primers were designed based on sequence homology information among mouse, rat, and human Cb28k cDNAs, and on the sequence of the partial hamster Cb28k cDNA that was identified by differential display. A 1423 bp full-length hamster calbindin-D28K cDNA was successfully cloned using total RNA isolated from untreated control hamster kidney by RT-PCR with primers 5′-CTC TCA AAC TAG CCG CTG CAC C-3′ (forward) and 5′-GAC AGA TTT CTG TGT CAC CTG TCT CCC TTC-3′ (reverse) (Fig. 1). This 1423 bp cDNA fragment was cloned into an expression vector, and the subsequently amplified and purified plasmid DNA was analyzed for the presence of the expected size fragment. Plasmid containing the appropriately sized insert was subjected to DNA sequence analysis using both M13 forward and reverse primers. Nested primers, whose sequence was deduced based on data obtained on previous round of sequencing with M13 forward and reverse primers were also used to sequence the clones, in order to attain complete complementarity and overlap of DNA bases. Based on the DNA sequence information, a full-length 1423 bp hamster Cb28k cDNA sequence was derived that shows an ATG start site and a TGA termination signal (Fig. 2). This 1423 bp gene contains a 786 bp open reading frame, which corresponds to a functional protein consisting of 261 amino acids (Fig. 2) with a predicted molecular mass of 28 kDa. Full-sequence homology queries sought using the NCBI BLAST Database showed close matches to rat, mouse, and human Cb28k nu-

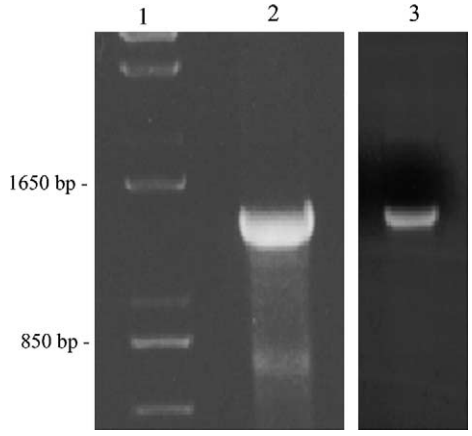

Fig. 1. PCR amplification of calbindin cDNA. Total RNA from male Syrian hamster kidney was reverse transcribed and used for PCR amplification. Primer set 5′-CTC TCA AAC TAG CCG CTG CAC C (forward) and 5′-GAC AGA TTT CTG TGT CAC CTG TCT CCC TTC (reverse) resulted in the amplification of a 1423 bp cDNA fragment (lane 2). This fragment was gel purified (lane 3) and subjected to DNA sequence analysis. 1 = 100 bp DNA ladder.

cleotide and amino acid sequences (Table 1). The hamster Cb28k cDNA sequence information has been deposited with the gene bank (Accession No. AY485347).

3.2. mRNA analysis of Cb28k

Messenger RNA levels of calbindin were measured in untreated age-matched control kidney tissues of hamsters and in E2-induced renal tumor tissue by RT-PCR analysis. The PCR-amplified cDNA was size fractionated on agarose gels and visualized by ethidium bromide staining. The intensity of the PCR-generated bands was measured using a Molecular Dynamics densitometer/ImageQuaNT program (Sunnyvale, CA), and the raw data was converted to a ratio of calbindin to cyclophilin band intensity. About 90% decrease in the Cb28k mRNA levels was detected in the E2-induced kidney tumors compared with untreated controls (Fig. 3).

Table 1
Percentage nucleotide (nt) and deduced amino acid (aa) homology between hamster calbindin-D28K cDNA and mouse, rat, and human calbindin-D28K cDNA

	Rat	Mouse	Human
Percentage identity—nt	94	95	93
Percentage identity—aa	98	98	98

3.3. Immunoblot analysis for Cb28k protein

Western blot analysis was performed on proteins isolated from untreated age-matched control kidneys of hamsters and from E2-induced renal tumor tissue in order to analyze the expression of Cb28k at the protein level. About 75% decrease in the protein levels of Cb28k was detected in renal tumor tissue of E2-treated hamsters compared with untreated controls (Fig. 4).

4. Discussion

In the present study, we report the cloning of hamster Cb28k cDNA and demonstrate the suppression of both

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ctctcaaactagccgctgcacc atg gca gaa tcg cac cta cag tca tcc ctg atc aca gcc tca cag ttt ttc gag atc
                        M  A  E  S  H  L  Q  S  S  L  I  T  A  S  Q  F  F  E  I
tgg ctt cat ttc gac gct gac gga agt ggt tac ctg gaa gga aag gag ctg cag aac ttg atc cag gag ctc ctg
 W  L  H  F  D  A  D  G  S  G  Y  L  E  G  K  E  L  Q  N  L  I  Q  E  L  L
cag gcg cga aag aag gct gga ttg gag tta tca cct gag atg aaa acc ttt gtg gat cag tat ggg cag aga gat
 Q  A  R  K  K  A  G  L  E  L  S  P  E  M  K  T  F  V  D  Q  Y  G  Q  R  D
gat ggc aaa ata gga att gta gag ctg gct cat gtc ttg ccc aca gaa gag aat ttc ctg ctg ctg ttc cga tgc cag
 D  G  K  I  G  I  V  E  L  A  H  V  L  P  T  E  E  N  F  L  L  L  F  R  C  Q
caa ctg aag tcc tgt gag gaa ttt atg aag act tgg agg aag tat gac act gac cac agc ggc ttc ata gaa act
 Q  L  K  S  C  E  E  F  M  K  T  W  R  K  Y  D  T  D  H  S  G  F  I  E  T
gag gaa ctt aag aac ttt cta aag gac ctg cta gag aaa gcc aac aag act gtg gat gat aca aaa cta gct gag
 E  E  L  K  N  F  L  K  D  L  L  E  K  A  N  K  T  V  D  D  T  K  L  A  E
tac aca gac ctc atg ctg aaa cta ttt gat tca aat aat gat gga aag ctg gaa ctg aca gag atg acc agg tta cta
 Y  T  D  L  M  L  K  L  F  D  S  N  N  D  G  K  L  E  L  T  E  M  T  R  L  L
cca gtg cag gaa aat ttc ctt ctt aaa ttc cag gga atc aaa atg tgt ggg aaa gag ttc aat aag gct ttt gag tta
 P  V  Q  E  N  F  L  L  K  F  Q  G  I  K  M  C  G  K  E  F  N  K  A  F  E  L
tat gat cag gat ggc aac gga tac ata gat gaa aat gag ctg gat gct tta ctg aag gat ctg tgt gag aag aac
 Y  D  Q  D  G  N  G  Y  I  D  E  N  E  L  D  A  L  L  K  D  L  C  E  K  N
aaa cag gaa ctg gat gtt aat aat att act aca tac aag aag aac ata atg gcc ttg tcg gat gga ggg aag ctg tac
 K  Q  E  L  D  V  N  N  I  T  T  Y  K  K  N  I  M  A  L  S  D  G  G  K  L  Y
cga aca gat ctt gcc ctt att ctc tct gct ggg gac aac tag ttgtatctaaaacataactgtgcgctataaaagagtaggctgta
 R  T  D  L  A  L  I  L  S  A  G  D  N  *
ttttcttttatatctgtaaattctactgcatatagagaattatccaggatgcgttacacattcttttctgcttgtttctatactgtttgtaatgtacagttttt
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Fig. 2. Nucleotide sequence analysis and putative amino acid sequence of hamster calbindin D28K.

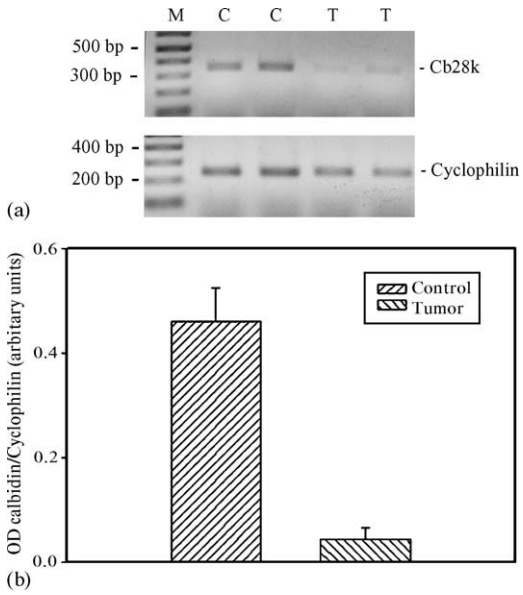

Fig. 3. Differential expression of Cb28k mRNA. Male Syrian hamsters were treated with 25 mg pellets of E2 for 7 months. Control animals were sham operated and left untreated. Total RNA from E2-induced renal tumors (T) and from kidneys of age-matched sham operated control hamsters (C) was isolated and reverse transcribed, and both Cb28k and cyclophilin cDNAs were PCR amplified. The PCR amplified products are resolved on 1.5% agarose gels and visualized by ethidium bromide staining (a). Negative films of ethidium bromide stained gels were quantified using a Molecular Dynamics densitometer/ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). About 90% decrease in Cb28k mRNA levels was detected in E2-induced renal tumors (T) compared with sham-operated controls (b). The data presented are a ratio of the OD of Cb28k to cyclophilin band intensity and are a mean ± S.E. of four different RT-PCR experiments performed using tissues obtained from four different animals in each group.

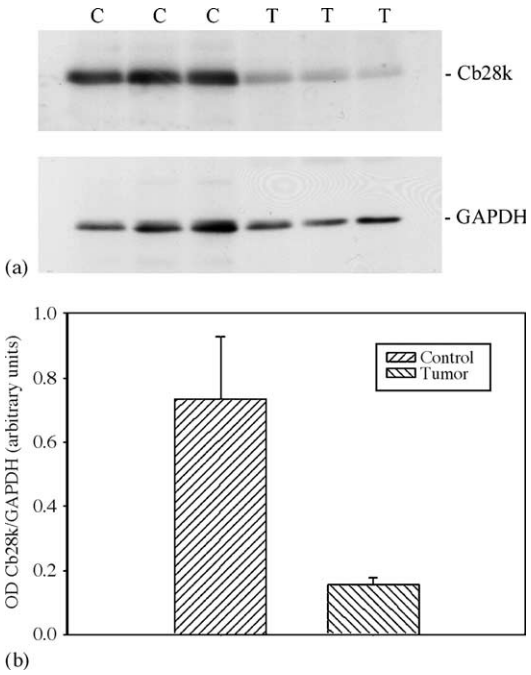

Fig. 4. Western blot immunodetection of calbindin protein. Male Syrian hamsters were treated with 25 mg pellets of E2 for 7 months. Control animals were sham operated and left untreated. Total protein was isolated from renal tumors and from kidneys of age-matched sham operated hamsters. An affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of Cb28k of human origin was used for immunodetection. C = age-matched control kidney tissue, T = estrogen-induced renal tumor tissue (a). Each lane contains 20 μg of total protein. The same membrane was stripped and reincubated with glyceraldehyde-3-phoshate dehydrogenase (GAPDH) monoclonal mouse anti-rabbit antibody (b). Semiquantitative analysis was performed by using a Molecular Dynamics densitometer/ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA), which demonstrated about 75% decrease in Cb28k protein level in E2-induced tumors compared with untreated controls (b) The data presented are a mean ± S.E. of the OD of three different protein samples obtained from three different animals in each group and are represented as a ratio of Cb28k/GAPDH.

Cb28k mRNA and protein in E2-induced hamster kidney tumors. The 1423 bp hamster Cb28k cDNA contains an open reading frame putatively translated into a 261 amino acid long peptide that shares high nucleotide and amino acid sequence homology with mouse, rat, and human Cb28k cDNAs (Table 1). Though its nucleotide sequence is 95% matched to mouse, 94% to rat, and 93% to human Cb28k mRNA, the amino acid sequence of the hamster Cb28k protein bears 98% homology to its mouse, rat, and human counterparts [33–35]. Several functional domains, including the Ca^{2+}-binding domain (EF-Hand super family), are conserved across the four species. There is one amino acid difference between hamster and mouse (V228I hamster → mouse), two amino acid differences between hamster and rat (V228I, T232S hamster → rat), and three amino acid differences between hamster and human (L44Q, E225D, and V228I hamster → human) Cb28k proteins. The V228I, T232S and E225D substitutions are conservative in the amino acid properties, and so it would be unlikely that they would result in significant differences in protein function. However, whereas the coding sequence of Cb28k is highly conserved across different species, the 3′-untranslated region of calbindin-D28k cDNA shows a much greater degree of variability, which may suggest differential degree of regulation.

Cb28k is a Vitamin D3-dependent calcium binding protein that is localized to a variety of tissues, including brain, kidney, intestine, lung, and reproductive organs [21]. It is a phosphoprotein that may be regulated via a protein kinase C signal transduction pathway, can bind three to five calcium ions, and may act as a buffer to maintain intracellular calcium homeostasis [22], but its exact role is still unknown. It has recently been shown that Cb28k, a target of Vitamin D action, is present in osteoblasts and protects against TNF- and glucocorticoid-induced apoptosis of osteoblastic cells [36]. Cb28k gene also seems to be regulated in aging and aging-related neurodegenerative diseases [37,38]. Cb28k transcripts are 50–80% less in abundance in the hamster cerebellum of aged animals, compared with younger animals, suggesting that this calcium binding protein may also play an important role in neuronal degeneration [37]. A selective

and substantial age-related loss of Cb28k has been demonstrated in cholinergic neurons of the basal forebrain, which are selectively vulnerable in neurodegenerative disorders of the elderly, particularly in Alzheimer's disease [38]. A tissue- and organ-specific regulation of Cb28k gene expression by estradiol has been demonstrated. In mouse models, calbindin-D28k gene expression is down-regulated by estradiol in the uterus, but not in ovaries and oviduct [39,40].

Oxidative stress induces cellular damage to lipids, proteins, and membranes, and the oxidative stress created by metabolic redox cycling between catechol estrogens and estrogen quinones has been suggested to play an important role in the estrogen-induced carcinogenic processes [7,9,20,41]. Although the concentration of the unconjugated catechol estrogens is very low in systemic circulation [42,43], there are other factors that contribute to the build up of catechol estrogens in the target tissue. For example, 2-hydroxyestradiol inhibits the methylation of 4-hydroxyestradiol [44–46], thus making it available for a longer time for redox cycling and subsequent generation of oxidative stress. 4-Hydroxyestradiol is a carcinogenic metabolite of estradiol in rodent models [47,48]. Moreover, a temporary decrease in quinone reductase activity has been reported in estrogen-treated hamster kidney, which may enhance the formation of free radical intermediates and oxidative stress during the biotransformation of estrogens [49]. Cb28k has been associated with greater resistance to oxidative stress [23,24]. In addition, free radical formation by cytokines is inhibited in Cb28k transfected pancreatic beta cells [50]. Because of the suppression of Cb28k in E2-induced tumors, the preventative or protective effects of Cb28k against oxidative stress may not be available. Thus, this gene may have a major role in protecting against free radicals, oxidative stress, and cellular degeneration in different cell types. In addition, transfection of Cb28k gene into the murine proximal tubular epithelial cells has been shown to serve a protective function against chemical hypoxic injury [51], and Cb28k protein overexpression has been shown to protect neurons against focal cerebral ischemia [52].

Although the exact role of Cb28k in carcinogenesis is not known, studies of human lung carcinomas, and adenocarcinomas in particular, demonstrate that patients with Cb28k-positive tumors had better overall survival than patients whose tumors were Cb28k-negative [53]. The percentage of Cb28k containing tissues in small cell lung cancer is significantly higher than in non-small cell lung cancer, while the Cb28k concentration is low in normal lung extracts [54]. This may be because of the protective anti-oxidant effect of Cb28k. In general, Cb28k seems to be present in subpopulations of neuroendocrine phenotypes in midgut and foregut carcinoids, and in small-cell carcinomas, but surprisingly, it is not found in adenocarcinomas, leiomyomas/leiomysarcomas, schwannomas, and lymphomas, which suggests that Cb28k is possibly a novel adjuvant neuroendocrine marker that can potentially be useful in diagnostic tumor immunohistochemistry [55].

In summary, estrogen use has been associated with the development of human breast and uterine tumors [1,2]. E2, in particular, induces high levels of oxidative stress in vitro and in vivo systems [7,17], which greatly contributes to the carcinogenic potential of this chemical [16–20]. Cb28k gene has been indicated to provide a cellular defense system against oxidative stress [23,24]. Cb28k may also play an important role in protecting cells and tissues against oxidative stress mediated damage, and it may be an important biomarker in the situation where oxidative stress/free radicals are the major contributors to the carcinogenic process. Further studies need to be performed to elucidate the protective or preventative effect of Cb28k against oxidative stress.

Acknowledgements

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References

[1] P.G. Toniolo, M. Levitz, A. Zeleniuch-Jacquotte, S. Banerjee, K.L. Koenig, R.E. Shore, P. Strax, B.S. Pasternack, A prospective study of endogenous estrogens and breast cancer in postmenopausal women, J. Natl. Cancer Inst. 87 (1995) 190–197.

[2] J.L. Kelsey, M.D. Gammon, E.M. John, Reproductive factors and breast cancer, Epidemiol. Rev. 15 (1993) 36–47.

[3] The Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52705 women with breast cancer and 108411 women without breast cancer, Lancet 350 (1997) 1047–1059.

[4] Report on Carcinogens, tenth ed., U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program, December 2002.

[5] Writing Group for the Women's Health Initiative Investigators, Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial, JAMA 288 (2002) 321–333.

[6] H. Kirkman, Estrogen-induced tumors of the kidney. Growth characteristics in the Syrian hamster, Natl. Cancer Inst. Monogr. 1 (1959) 1–57.

[7] H.K. Bhat, G. Calaf, T.K. Hei, T. Loya, J.V. Vadgama, Critical role of oxidative stress in estrogen-induced carcinogenesis, Proc. Natl. Acad. Sci. USA 100 (2003) 3913–3918.

[8] J.D. Shull, T.J. Spady, M.C. Snyder, S.L. Johansson, K.L. Pennington, Ovary-intact but not ovariectomized female ACI rats treated with 17beta-estradiol rapidly develop mammary carcinoma, Carcinogenesis 18 (1997) 1595–1601.

[9] B.T. Zhu, D. Roy, J.G. Liehr, The carcinogenic activity of ethinyl estrogens is determined by both their hormonal characteristics and their conversion to catechol metabolites, Endocrinology 132 (1993) 577–583.

[10] X. Han, J.G. Liehr, 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis, Cancer Res. 54 (1994) 5515–5517.

[11] T. Tsutsui, Y. Tamura, E. Yagi, J.C. Barrett, Involvement of genotoxic effects in the initiation of estrogen-induced cellular transformation: studies using Syrian hamster embryo cells treated with 17β-estradiol and eight of its metabolites, Int. J. Cancer. 86 (2000) 8–14.

[12] J.G. Liehr, D.A. Sirbasku, Estrogen-dependent kidney tumors, in: M. Taub (Ed.), Tissue Culture of Epithelial Cells, Plenum Press, New York, NY, 1985, pp. 205–234.
[13] J.G. Liehr, Hormone-associated cancer: mechanistic similarities between human breast cancer and estrogen-induced kidney carcinogenesis in hamsters, Environ. Health Perspect. 105 (1997) 565–569.
[14] J.J. Li, S.A. Li, T.D. Oberley, J.A. Parson, Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: relation to hormonal activity and cell proliferation, Cancer Res. 55 (1995) 4347–4351.
[15] J.G. Liehr, G.M. Stancel, L.P. Chorich, G.R. Bousfield, A.A. Ulubelen, Hormonal carcinogenesis: separation of estrogenicity from carcinogenicity, Chem. Biol. Interact. 59 (1986) 173–184.
[16] A.A. Yuzpe, Oral contraception: trends over time, J. Reprod. Med. 47 (2002) 967–973.
[17] M.M. Patel, H.K. Bhat, Differential oxidant potential of carcinogenic and weakly carcinogenic estrogens: involvement of metabolic activation and cytochrome P450, J. Biochem. Mol. Toxicol. 18 (2004) 37–42.
[18] B.T. Zhu, A.H. Conney, Functional role of estrogen metabolism in target cells: review and perspectives, Carcinogenesis 19 (1998) 1–27.
[19] J.G. Liehr, D. Roy, Free radical generation by redox cycling of estrogens, Free Radic. Biol. Med. 8 (1990) 415–423.
[20] J.D. Yager, Endogenous estrogens as carcinogens through metabolic activation, J. Natl. Cancer Inst. Monogr. 27 (2000) 67–73.
[21] S. Chistakos, C. Gabrielides, W.B. Rhoten, Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations and molecular biology, Endocr. Rev. 10 (1989) 3–26.
[22] A. Gagnon, J. Welsh, Modulation and phosphorylation of calbindin-D28K correlates with protein kinase C activation 1, Biochem. Cell. Biol. 75 (1997) 33–40.
[23] J. Hugon, F. Hugon, F. Esclaire, M. Lesort, A.G. Diop, The presence of calbindin in rat cortical neurons protects in vitro from oxidative stress, Brain Res. 29 (1996) 288–292.
[24] Q. Guo, S. Christakos, N. Robinson, M.P. Mattson, Calbindin-D28k blocks the proapoptotic actions of mutant presenilin 1: reduced oxidative stress and preserved mitochondrial function, Proc. Natl. Acad. Sci. USA 95 (1998) 3227–3232.
[25] H.K. Bhat, H.J. Hacker, P. Bannasch, E.A. Thompson, J.G. Liehr, Localization of estrogen receptors in interstitial cells of hamster kidney and in estradiol-induced renal tumors as evidence of the mesenchymal origin of this neoplasm, Cancer Res. 53 (1993) 5447–5451.
[26] P. Chomzynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[27] L.M. Everett, A. Li, G. Devaraju, A. Caperell-Grant, R.M. Bigsby, A novel estrogen-enhanced transcript identified in the rat uterus by differential display, Endocrinology 138 (1997) 3836–3841.
[28] P. Liang, A.B. Pardee, Differential display of eukaryotic messenger RNA by means of polymerase chain reaction, Science 257 (1992) 967–971.
[29] S.P. Bhat, DNA sequencing on minigels: an alternative technique for routine analysis, Anal. Biochem. 212 (1993) 560–562.
[30] M. McClelland, K. Chada, J. Welsh, D. Ralph, Arbitrary primed PCR fingerprinting of RNA applied to mapping differentially expressed genes, EXS 67 (1993) 103–115.
[31] H.K. Bhat, J.V. Vadgama, Hamster estrogen receptor cDNA: cloning and mRNA expression, J. Steroid Biochem. Mol. Biol. 72 (2000) 47–53.
[32] H.K. Bhat, W.R. Hiatt, C.R. Hoppel, E.P. Brass, Skeletal muscle mitochondrial DNA injury in patients with unilateral peripheral arterial disease, Circulation 99 (1999) 807–812.
[33] N. Lomri, C. Perret, N. Gouhier, M. Thomasset, Cloning and analysis of calbindin-D28K cDNA and its expression in the central nervous system, Gene 80 (1989) 87–98.
[34] W. Hunziker, S. Schrickel, Rat brain calbindin D28: six-domain structure and extensive amino acid homology with chicken calbindin D28, Mol. Endocrinol. 2 (1988) 465–473.
[35] T.L. Wood, Y. Kobayashi, G. Frantz, S. Varghese, S. Cristakos, A.J. Tobin, Molecular cloning of mammalian 28,000 Mr Vitamin D-dependent calcium binding protein (calbindin-D28K): expression of calbindin-D28K RNAs in rodent brain and kidney, DNA 7 (1988) 585–593.
[36] P.R. Turner, S. Mefford, S. Christakos, R.A. Nissenson, Apoptosis mediated by activation of the G protein-coupled receptor for PTH/PTHrP in UMR106 osteoblastic cells, Am. J. Physiol. Endocrinol. Metab. 281 (2000) E162–E170.
[37] J. Kishimoto, T. Tsuchiya, H. Cox, P.C. Emerson, Y. Nakayama, Age-related changes of calbindin-D28k, calretinin, and parvalbumin mRNAs in the hamster brain, Neurobiol. Aging 19 (1998) 77–82.
[38] C. Geula, J. Bu, N. Nagykery, L.F. Scinto, J. Chan, J. Joseph, R. Parker, C.K. Wu, Loss of calbindin-D28k from aging human cholinergic basal forebrain: relation to neuronal loss, J Comp. Neurol. 455 (2003) 249–259.
[39] L.A. Opperman, T.J. Saunders, D.E. Bruns, J.C. Boyd, S.E. Mills, M.E. Bruns, Estrogen inhibits calbindin-D28k expression in mouse uterus, Endocrinology 130 (1992) 1728–1735.
[40] R.K. Gill, S. Christakos, Regulation by estrogen through the 5′-flanking region of the mouse calbindin-D28k gene, Mol. Endocrinol. 9 (1995) 319–326.
[41] J.G. Liehr, Is estradiol a genotoxic mutagenic carcinogen? Endocr. Rev. 21 (2000) 40–54.
[42] M.B. Lipsett, G.R. Merriam, S. Kono, D.D. Brandon, D.G. Pfeiffer, D.L. Loriaux, Metabolic clearance of catechol estrogens, in: G.R. Merriam, M.B. Lipsett (Eds.), Catechol Estrogens, Raven Press, New York, NY, 1983, pp. 105–114.
[43] G. Emons, G.R. Merriam, D. Pfeiffer, D.L. Loriaux, P. Ball, R. Knuppen, Metabolism of exogenous 4- and 2-hydroxyestradiol in the human male, J. Steroid Biochem. 28 (1987) 499–504.
[44] J. Weisz, Q.D. Bui, D. Roy, J.G. Liehr, Elevated 4-hydroxylation of estradiol by hamster kidney microsomes: a potential pathway of metabolic activation of estrogens, Endocrinology 131 (1992) 655–661.
[45] B.T. Zhu, J.G. Liehr, Inhibition of the catechol-*O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by catecholamine: implications for the mechanism of estrogen-induced carcinogenesis, Arch. Biochem. Biophys. 304 (1993) 248–256.
[46] D. Roy, J. Weisz, J.G. Liehr, The *O*-methylation of 4-hydroxyestradiol is inhibited by 2-hydroxyestradiol: implications for estrogen-induced carcinogenesis, Carcinogenesis 11 (1990) 459–462.
[47] J.G. Liehr, W.F. Fang, D.A. Sirbasku, A. Ari-Ulubelen, Carcinogenicity of catechol estrogens in Syrian hamsters, J. Steroid Biochem. 24 (1986) 353–356.
[48] R.R. Newbold, J.G. Liehr, Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens, Cancer Res. 60 (2000) 235–237.
[49] D. Roy, J.G. Liehr, Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male Syrian hamsters. Increased superoxide formation by redox cycling of estrogen, J. Biol. Chem. 263 (1988) 3646–3651.
[50] A. Rabinovitch, W.L. Suarez-Pinzon, K. Sooy, K. Strynadka, S. Christakos, Expression of calbindin-D(28k) in a pancreatic islet beta-cell line protects against cytokine-induced apoptosis and necrosis, Endocrinology 142 (2001) 3649–3655.
[51] M.J. Wu, L.W. Lai, Y.H. Lien, Cytoprotective effects of calbindin-D28k against antimycin-A induced hypoxic injury in proximal tubular cells, Life Sci. 71 (2002) 559–569.
[52] M.A. Yenari, M. Minami, G.H. Sun, T.J. Meier, D.M. Kunis, J.R. McLaughlin, D.Y. Ho, R.M. Sapolsky, G.K. Steinberg, Calbindin-D28k overexpression protects striatal neurons from transient focal cerebral ischemia, Stroke 32 (2001) 1028–1035.

[53] C.Y. Castro, M. Stephenson, M.M. Gondo, L.J. Medeiros, P.T. Cagle, Prognostic implications of calbindin-D28k expression in lung cancer: analysis of 452 cases, Mod. Pathol. 13 (2000) 808–813.
[54] H. Watanbe, M. Imaizumi, T. Ojika, T. Abe, T. Hida, K. Kato, Evaluation of biological characteristics of lung cancer by the human 28 kDa Vitamin D-dependent calcium binding protein, calbindin-D28k, Jpn. J. Clin. Oncol. 24 (1994) 121–127.
[55] C.D. Katsetos, M.M. Jami, L. Krishna, R. Jackson, A.S. Patchefsky, H.S. Cooper, Novel immunohistochemical localization of 28,000 molecular weight (Mr) calcium binding protein (calbindin-D28k) in enterochromaffin cells of the human appendix and neuroendocrine tumors (carcinoids and small-cell carcinomas) of the midgut and foregut, Arch. Pathol. Lab. Med. 118 (1994) 633–639.